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Expression and Characterization of Biologically Active Human Extracellular Superoxide Dismutase in Milk of Transgenic Mice*

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We have targeted the expression of recombinant human extracellular superoxide dismutase, a glycosylated tetrameric metalloprotein, to the mammary gland of transgenic mice. This was achieved by using regulatory elements from either the murine whey acidic protein gene or the ovine β-lactoglobulin gene to control expression of human extracellular superoxide dismutase cDNA. Whey acidic protein regulatory sequences directed high level mammary gland-specific expression of the recombinant gene and secretion of biologically active extracellular superoxide dismutase into the milk. The produced recombinant protein was fully active, it was in tetrameric form, it showed heparin affinity, and its mass was similar to that of the native enzyme. In addition, the *in vivo* plasma clearance in a rabbit model was similar to the previously studied native and recombinant forms. To our knowledge, this is the first example of efficient production of a tetrameric, protease-susceptible metalloprotein in milk of transgenic animals. Production at equivalent levels in transgenic farm animals would yield sufficient extracellular superoxide dismutase for therapeutic purposes.

The secretory extracellular superoxide dismutase (EC-SOD)[1] is one of three different SOD isoenzymes present in mammals (1). It is the major SOD isoenzyme in plasma, lymph, and synovial fluid (2–5) but exists primarily in the interstitial space of tissues (6–8). The other two isoenzymes are the dimeric CuZn-SOD, which is an intracellular cytosolic enzyme, and the tetrameric Mn-SOD, which is found in the mitochondrial matrix (9–11). The human EC-SOD cDNA has been isolated and sequenced (12), and the recombinant protein has been produced in mammalian cells (13). The protein is composed of four equal subunits, each containing 222 amino acids with a calculated molecular mass of 24.2 kDa (13). Each subunit binds one copper and one zinc atom and has a single *N*-glycosylation site at asparagine 89 (13, 14). The glycan of the native protein has not been the subject of any thorough investigation, but its lectin binding properties have been studied (1, 13). A fundamental and distinguishing property of EC-SOD is its affinity for some glycosaminoglycans such as heparin and heparan sulfate (1, 3, 4, 15, 16). The latter, which exists in the glycocalyx of cell surfaces and in the connective tissue matrix, is the important physiological ligand of EC-SOD. Because of this affinity, EC-SOD forms in the vasculature an equilibrium between the plasma phase and the glycocalyx of the endothelium (3, 4, 17, 18). In tissues, virtually all EC-SOD exists anchored to heparan sulfate in the interstitial space and on the cell surfaces (8). The positively charged carboxyl-terminal end of the enzyme is responsible for the heparin binding (19). This domain is sensitive to proteolysis (20) resulting in EC-SOD forms with reduced or absent heparin affinities *in vivo* (8).

Upon intravenous injection, EC-SOD rapidly binds to the surface of the endothelium and displays a long half-life (15–20 h) in the vasculature (15, 18). CuZn-SOD (parenterally administered) has been shown to exert a host of therapeutic actions (21–25). The experience with EC-SOD is in comparison small (26–33), but this isoenzyme appears even more potent. The high efficacy is most certainly related to the special pharmacokinetic properties of the enzyme.

Genetic control elements from different milk protein genes have been used to direct production of recombinant proteins in the milk of transgenic animals (34–36). The use of such transgenic techniques in farm animals offers the potential to produce pharmaceutical proteins in the milk. The potential advantages are the capability to produce recombinant, complex post-translationally modified, human proteins in large quantities in a highly cost-effective system without detriment to the producing animal.

The temporal and spatial distribution of recombinant gene expression directed by milk protein gene regulatory fragments from various species seems to be very conserved and general among all species studied (37, 38). Despite the fact that many cDNAs and genomic fragments now have been evaluated in mammary gland expression systems in transgenic animals, it is hard to define the factors that affect the level of expression. The expression levels reported are for some vector constructs very high, whereas they are disappointingly low for others (35). The qualitative aspects related to detailed analysis of post-translational modifications of recombinant proteins produced in the milk of transgenic animals have received limited attention. In this study, the expression and functionality of a recombinant human glycosylated multimeric metalloprotein expressed in mouse milk are described.

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[1] The abbreviations used are: EC-SOD, extracellular superoxide dismutase; SOD, superoxide dismutase; bp, base pair(s); WAP, whey acidic protein; kb, kilobase pair(s); MOPS, 3-(*N*-morpholino)propanesulfonic acid; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis.

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...LS AND METHODS

...loc... in t... ...e ... located about 1.6 kb ... last exon was i... ig. ...).

Tw... g... WAP/EC... ...ome anim... 178 in- jec... ...ated in... 7 fo... mott... ...ing in ... e. 14 animals were ... ...arp... WA... ...mor... analys... ...ity of the injected were confirm... ...al analysis (Fig. 1B). of t... f... ...ales, and two w... One ... ...fore it was bred. Fo... anim... ...ing fem...les were generat... all ... ...s of this WAP ... construc... ...vector contains a long ... ...segment of the 3.8-kb, in addition, the f... ... of EC-SOD was inserted directly downstr... ...ein of WAP exon 1. This ... ... of the translation start ... harboring this vector was

The β-lactoglobulin/EC-SOD construct ... 160 ova that were injected into 16 f... ... in the birth of ... ice. When analyzing t... ..., the trans- genic mi... ...ti...d and analyzed for expressi... recombinant p...

...restr... ...the suc... ...levels of t... ...minant

Mg Li Ki Sp He La

4.4 –

2.4 –

1.4 –

A

1 kb

EcoRI XhoI KpnI SalI PstI PstI EcoRI

I III

WAP hEC-SOD

B

1 2 3 4 5 6 7 8 9 10 11 12 13 14

3.0 –

1.6 –

1.0 –

0.5 –

b 1 2

4.4 –

2.4 –

1.4 –

...IG. 1. ...ture of th... ...on ... ...AP/... ...-SOD ...

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TABLE I
Production of human EC-SOD in milk of transgenic mice

Vector	Transgenic line	EC-SOD
		ng/ml
[illegible]2	[illegible]	90
		UD[a]
		710,000
		15
[illegible]	[illegible]02	UD
	[illegible]	8
None	Control	0–0.76

[a] UD, u[illegible], within the range of controls.

al[illegible] ain. In additional experiments, where the same [illegible] ory sequences were used to direct expression of [illegible] DNAs, no recombinant gene expression was [illegible] in.[2]

[illegible] f mammary gland RNA of [illegible] e lines 68 and [illegible] hybridized with a mouse WAP exon [illegible] and exon [illegible], which hybridizes to the same extent to both endogenous WAP mRNA and to recombinant WAP/EC-SOD [illegible]. The endogenous WAP mRNA level was found to be significantly higher than the level of recombinant WAP/EC-SOD mRNA (Fig. 2b). The β-lactoglobulin/EC-SOD transgenic lines were not analyzed for mRNA expression.

Secretion of human EC-SOD into Mouse Milk. EC-SOD was [illegible] determined with enzyme-linked immunosorbent [illegible]. The production varied a lot between the transgenic lines [illegible] overall higher in the WAP transgenic lines (Table I). [illegible], was displayed very high [illegible] around 0.7 [illegible]. This level of EC-SOD corresponds to [illegible] 1% of total milk protein.

Recombinant EC-SOD activity of control milk was about [illegible] units/ml. Enhancements in activity in the milk samples with [illegible] fusion could not be distinguished with certainty [illegible]. In line 68 the SOD activity in the milk was [illegible] and the specific activity [illegible] was similar to that of native and recombinant EC-SOD (19). Production of recombinant [illegible]

[illegible] A. Edlund, T. Johansson, S. Marklund, S. Bromm, M. Stromqvist, and J. Tornell, unpublished observation.

[illegible] was [illegible] Milk from [illegible] and [illegible] was [illegible] and [illegible] at room temperature with [illegible] protease inhibitors [illegible] at room temperature with [illegible] reduced and [illegible]

Milk from [illegible] SOD produced in [illegible] Sepharose [illegible] protein [illegible] and [illegible] mass.

[illegible] from [illegible] the milk [illegible] SOD was [illegible] inhibitor [illegible] the [illegible] inhibitor [illegible] inhibitor [illegible] inhibitor

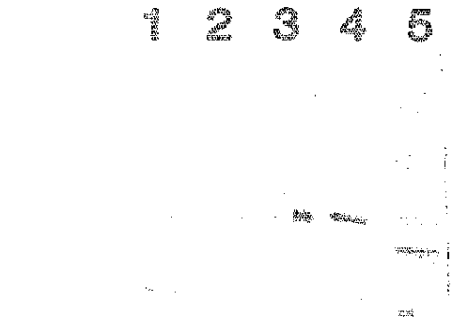

Fig. 4. ... of human EC-SOD ... from ... CLC ... and ... Methods. ... 9.5, 8.5, 27.5, and 18.5 kDa ... EC-SOD produced in CHO cells (25 ng), ... EC-SOD (170 ng), *lane 4*, recombinant EC-SOD produced ... line 68 mouse (85 ng). At each collection ... were added as described under "Methods," ... diluted 10 times, and the cases ... centrifugation and centrifugation.

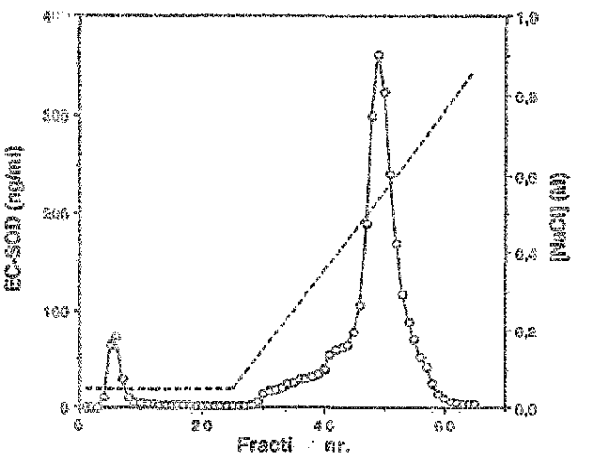

Fig. 5. ... Purified recombinant ... weight ... from line 68 ... about ... and separated on a ... Materials and Methods.

TABLE II

... Method of purification and recombinant human EC-SOD

... billed and calculated as EC-SOD/L culture. ... in the cells (CHO EC-SOD), and EC-SOD produced in cells ... EC-SOD). The fraction ... Fraction ... Gel ... Heparin ... Phenyl ...

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...	+	++	...	++	+
...	+	+	...	+	++

... elevated ...

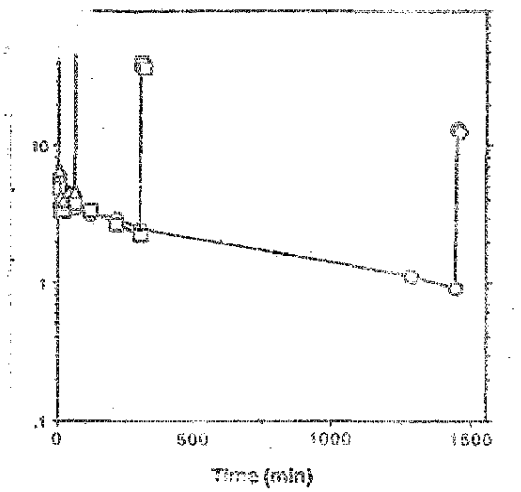

Fig. 6. Plasma ... of recombinant human EC-SOD ... in rats. The first ... of ... was ... in rats ... as shown in ... under "Experimental ... plasma concentration was ... the ... and ... n. ... were ... after 2, ... and ... h.

... at 1, 5, and 24 h led to ... This behavio... CHO-produced rec... CHO-produced EC-SOD displayed a ... circul... on (18).

DISCUSSION

... with ... Extracellular ... such ... ment ... This ... Ca/EC-SOD ... ide ... ment ... ed ... This ... ed ... ed. This ... ed ... ed ... ed

[illegible] finding does [illegible] ...ment EC-SODs contain oligosac... exist bound to EC-SOD in the ...mal ...saccharides are [illegible] by a sin...le ...OD stud... ...wever, EC-... ...by ...muscle cells,[3] a... poss... ...is well known that the ... ...tely when it is produced by ... ...human body (52).

...combinant EC-SOD from mouse m... ...initially to native and recombinant ..., after intravenous infus... ...rabbits. ...ion ... binding to the vasc... ...human as well as ...d release after injecti... ...n. ...lk-produced EC-SOD wa... ...e ...ly eliminated from the circulation than the ... ...studied here.

...conclude, our study shows that by u... ...enic ...ter... ...mammary gland can be used ... a bio... ...tor ...for ...production of biologically active hu... EC-SOD, a ...ric, protease-susceptible, copper- and zinc-containing glycoprotein.

...—We ... Jan-Olov Andersson, Åsa Skytt, and ... ...chnical assistance and Drs. Gunnar ... ...for ...erous support.

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